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Green tea catechins such as (−)-epicatechin and (−)-epigallocatechin accelerate Cu^{2+}-induced low density lipoprotein oxidation in propagation phase

Naoki Yamanaka*, Osamu Oda, Seiji Nagao

The Bio-Dynamics Research Institute, Nagoya Memorial Hospital, 4-305 Hirabari, Tenpaku-ku, Nagoya 468, Japan

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Abstract Effects of (−)-epicatechin (EC) and (−)-epigallocatechin (EGC) on Cu^{2+}-induced low density lipoprotein (LDL) oxidation were studied in initiation and propagation phases. When 1.5 μM EC or EGC was added to the mixture of isolated human LDL and Cu^{2+} in the initiation phase, the oxidation of LDL was inhibited in agreement with previous findings. In contrast, in the propagation phase, 1.5 μM of EC or EGC worked as an accelerator of the oxidation, and acceleration ratios (maximum about 6 times) were modified depending on the concentrations of catechin used and the oxidation process in the propagation phase. The evidence was obtained from formation of thiobarbituric acid reactive substances (TBARS), detecting conjugated diene measured by absorbance at 234 nm and investigating fragmentation of apoprotein B (apo B) in LDL. Even in the propagation phase of LDL oxidation, the elevated concentrations of EC or EGC worked as inhibitors: after 40 min incubation of LDL with Cu^{2+}, 10.0 μM EC or 2.0 μM EGC inhibited LDL oxidation. Yet, nitric oxide (NO) released from 5 μM zwitterionic polyamine/NO adducts had an inhibitory in all phases of LDL oxidation. These results indicate that catechins such as EC and EGC can act as free radical terminators (reducing agents) or accelerators (oxidizing agents) under oxidation circumstances, which is a different character from NO. From the above evidence, further investigations are needed on many natural flavonoids, the most potent antioxidative compounds in foods.

Key words: Catechin; Antioxidant; Oxidant; Low density lipoprotein

1. Introduction

Green tea leaves contain flavonoids known as catechins (flavan-3-ol derivatives), consisting of (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (+)-catechin ((+)C), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG) [1]. Catechins have been reported to have many pharmacological properties such as antimutagenic, anti-cancer promoting and hypolipidemic effects in addition to antioxidative effects and scavenging free radicals [1–7].

Oxidatively modified low density lipoprotein (LDL), which is taken up by scavenger receptors, is suggested to be involved in the development of atherosclerosis [8]. LDL oxidation can be catalyzed by heavy metals such as Cu^{2+} and Fe^{3+} [9,10], and promoted by activated cells [11,12]. Antioxidants should efficiently inhibit LDL oxidation and reduce the biological consequences such as uptake by macrophages. Probucol [4,13] and ascorbic acid [14] have been shown to protect oxidation of LDL or microsomes. Probucol was also reported to protect against spontaneous atherosclerosis in Watanabe heritable hyperlipidemic rabbits [15,16], and this report provides additional evidence that oxidized LDL is involved in the early stages of atherosclerosis.

The antioxidative effects of various catechins such as (+)C and EGCG on Cu^{2+}-induced LDL oxidation are of current interests in relation to the above described protection against the development of atherosclerosis [1–3]. Autoxidation of LDL is a free radical chain reaction and is separated into the processes of initiation, propagation and termination phase reactions [17].

Cu^{2+}-induced LDL oxidation processes were also subjected to rapid oxidation by the above mentioned chain reactions. In previous papers [1–3], the antioxidative effects of catechins on LDL oxidation were examined in the initiation phase. Therefore, in the present study, we investigated the effects of catechins such as EC and EGC in the propagation process of Cu^{2+}-induced LDL oxidation. We found that, depending on the concentrations used, EC and EGC had accelerative as well as inhibitory effects in the propagation phase of Cu^{2+}-induced LDL oxidation.

2. Materials and methods

2.1. Chemicals

EC, EGC, ECG, EGCG and (+)C were obtained from Kurita Kogyo Ltd. (Tokyo, Japan). 1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC5), which generates nitric oxide (NO), was from Dojin Chemicals Co., Ltd. (Kumamoto, Japan). Other chemicals were purchased from Wako Pure Chemicals (Tokyo, Japan).

2.2. Isolation of human LDL

Human plasma was obtained from healthy volunteers according to the Declaration of Helsinki. Human LDL ($d = 1.005$–1.065) was isolated from plasma by ultracentrifugation [18]. 1 mM EDTA was added to the plasma to prevent autoxidation of LDL. Isolated LDL was dialyzed against 20 mM sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride and 2 μM EDTA overnight at 4°C in the dark. Before use, the LDL solution was diluted with 0.1 M sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride and 0.2 μM EDTA (PBS-EDTA). The protein concentration of LDL was estimated with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as a standard.

2.3. LDL oxidation

LDL (0.2 mg protein/ml) in PBS-EDTA was incubated at 37°C in the presence of 5 μM $CuSO_4$ and the effects of various catechins were examined in the initiation, propagation and termination processes of LDL oxidation.

2.4. Measurement of LDL peroxidation

100 μl of sample, obtained from the reaction mixture every 20 min, was added to 20 μl of 10 mM EDTA to stop oxidation. Lipid peroxidation was estimated fluorometrically as thiobarbituric acid reactive substance (TBARS) formation [19] and/or measured by absorb-

*Corresponding author. Fax: (81) (52) 803-8830.

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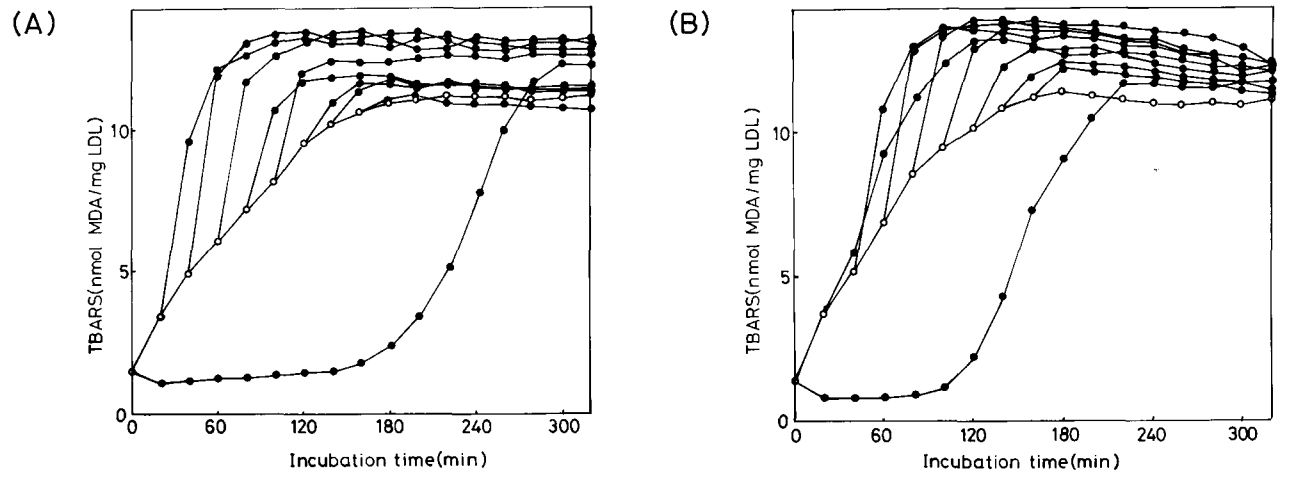


Fig. 1. Accelerative and inhibitory effects of EC and EGC on Cu^{2+}-induced TBARS formation in LDL. LDL (0.2 mg protein/ml) was dissolved in 25 ml PBS-EDTA and the reaction was initiated by the addition of 5 μM $CuSO_4$ at 37°C. A 2.0 ml aliquot was separated to add 1.5 μM EC or EGC at every 20 min. A 100 μl aliquot from each reaction mixture was obtained every 20 min to measure TBARS formation as described in Section 2. A: EC; B: EGC. ○: no addition; ●: addition of EC or EGC every 20 min during LDL oxidation.

ance at 234 nm to detect conjugated diene [20]. In TBARS analysis, malondialdehyde (MDA) formed from 1,1,3,3-tetraethoxypropane was used as a reference standard and the results were expressed as nmol equivalents of MDA. We have done these experiments two or three repeated times, and the results showed nearly the same tendency. Sample-to-sample variation from different volunteers was recognized. Therefore, typical results are shown in the figures.

2.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE for investigating fragmentation of apoprotein B (apo B) in LDL by oxidation was performed according to the method of Laemmli [21] using 4–12% gradient gel. 200 μl of reaction mixture was stopped by adding 50 μl of 10 mM EDTA. LDL protein was precipitated by 300 μl of 50% trichloroacetic acid solution and the resulting precipitated protein was collected by centrifugation at 10000×*g* for 20 min. The precipitate was washed 3 times with 200 μl of 10 mM phosphate buffer, pH 7.2, and lyophilized. Lyophilized samples were dissolved with 20 μl 10 mM Tris-HCl buffer, pH 7.2, containing 2% SDS and 8 M urea and 20 μg of protein was applied to SDS-PAGE.

3. Results

Fig. 1A shows the effects of addition of 1.5 μM EC every 20 min on 5 μM Cu^{2+}-induced LDL oxidation. It is evident that 1.5 μM EC accelerated TBARS formation in the propagation phase of LDL oxidation. In contrast, in the initiation phase, 1.5 μM EC (time of addition 0 min) inhibited LDL oxidation, in agreement with previous reports [1–3]. 1.5 μM EGC gave the accelerative and inhibitory effects of LDL oxidation in the propagation and initiation phases in a similar manner to EC (Fig. 1B). Other catechins such as (+)C, ECG and EGCG also had accelerative activities in the propagation phase (data not shown). The accelerative and inhibitory effects of 1.5 μM EC were confirmed by conjugated diene formation as shown in Fig. 2A. Fig. 3 shows the SDS-PAGE pattern of fragmentation of apo B under various conditions. As shown in Fig. 3A,

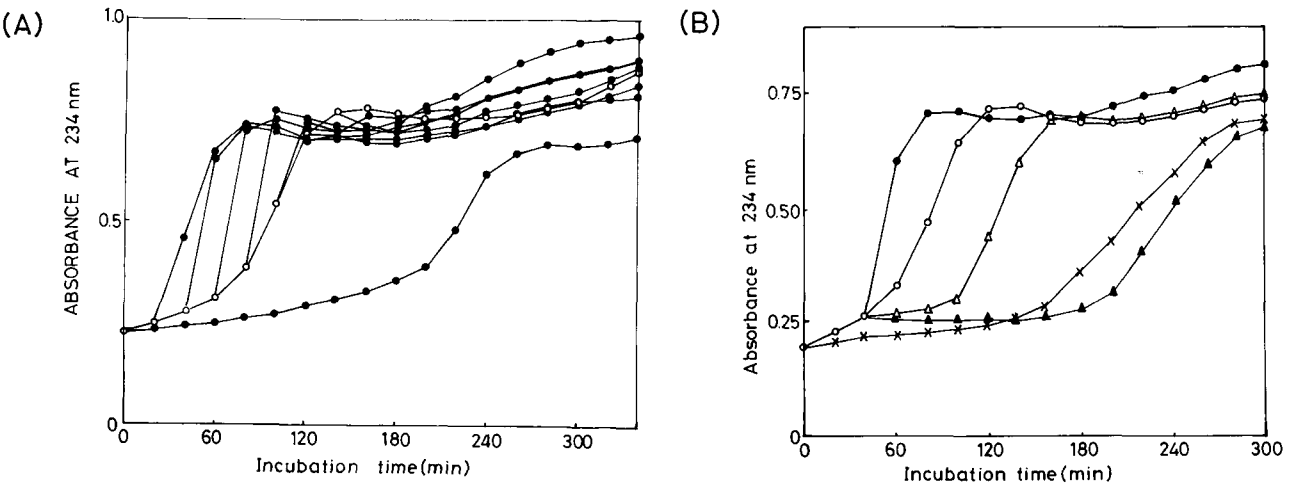


Fig. 2. Accelerative and inhibitory effects of EC and EGC on Cu^{2+}-induced conjugated diene formation in LDL. A: Effects of 1.5 μM EC in the initiation and propagation phases. LDL (0.2 mg protein/ml) was dissolved in 30 ml PBS-EDTA and the reaction was initiated by adding 5 μM $CuSO_4$ at 37°C. A 4.0 ml aliquot was separated to add 1.5 μM EC every 20 min. Conjugated diene formation was measured as described in Section 2. ○: no EC; ●: 1.5 μM EC. B: Effects of 1.5 μM and 5.0 μM EGC in the propagation phase and 5 μM NOC5 in the initiation and propagation phases. LDL (0.2 mg protein/ml) was dissolved in 20 ml PBS-EDTA and the reaction was initiated by adding 5 μM $CuSO_4$ at 37°C and conjugated diene was measured as control (○). A 4.0 ml aliquot was separated to add 5.0 μM NOC5 (×). After 40 min, a 4.0 ml aliquot was separated from the control solution, and 1.5 μM EGC (●), 5.0 μM EGC (△) or 5.0 μM NOC5 (▲) was added. Conjugated diene formation was measured as described in Section 2.

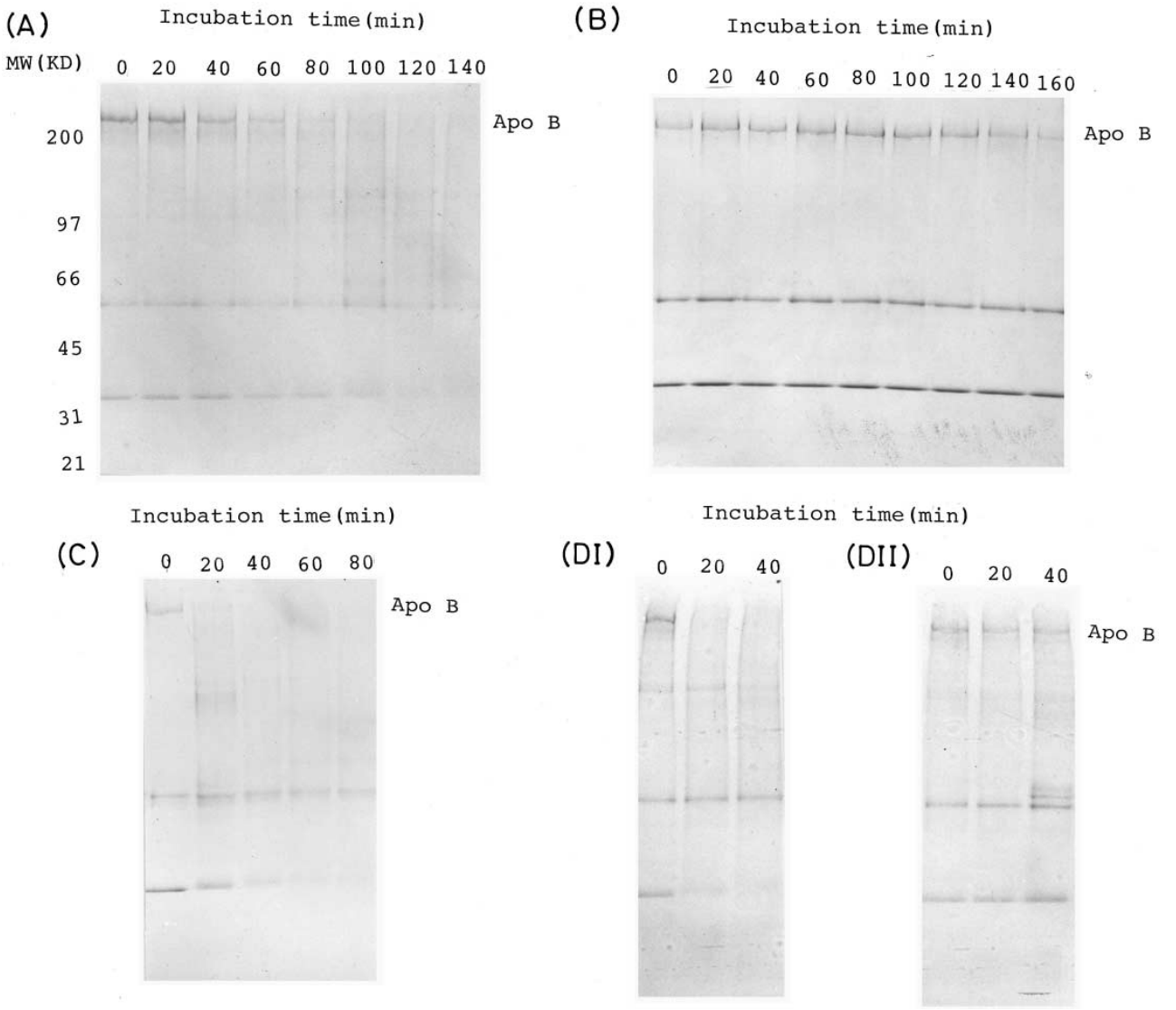


Fig. 3. Cu^{2+}-induced apo B fragmentation in the presence of EC and EGC. A: Apo B fragmentation during LDL oxidation with Cu^{2+}. 5 ml of LDL (0.2 mg protein/ml in PBS-EDTA) solution was incubated with 5 μM $CuSO_4$ at 37°C, designated R.M. After every 20 min incubation, 200 μl of the reaction mixture was removed to perform SDS-PAGE as described in Section 2. B: Effect of 1.5 μM EC in the initiation phase. EC (1.5 μM) was added at 0 min in 4.0 ml of R.M. The subsequent procedures were as described in A. C: Effect of 1.5 μM EC in the propagation phase. R.M. (2.0 ml) was incubated for 20 min at 37°C and 1.5 μM EC was added. Every 20 min, a 200 μl aliquot was removed to perform SDS-PAGE as described in A. D: Effects of 1.5 μM (I) and 5.0 μM EGC (II) in the propagation phase. After 40 min incubation of R.M. (5.0 ml), an aliquot (2.5 ml) was separated to add 1.5 μM or 5.0 μM EGC. After every 20 min with EGC, an aliquot (200 μl) was removed and the subsequent procedures were as described in A.

when LDL was incubated with 5 μM Cu^{2+} in the absence of catechin, the apo B band was clearly observed until 60 min, but disappeared thereafter. With addition of 1.5 μM EC at 0 min, the apo B band was detected even after 160 min incubation (Fig. 3B). Yet, when 1.5 μM EC was added after 20 min incubation of LDL with 5 μM Cu^{2+}, fragmentation of apo B was accelerated and the apo B band had disappeared 20 min after EC addition as shown in Fig. 3C. The fragmentation of apo B band was also accelerated to disappear 20 min the addition of 1.5 μM EGC (Fig. 3D-I). In contrast, 5.0 μM EGC protected the fragmentation (Fig. 3D-II).

The accelerative and inhibitory effects of EC and AGC were changed depending on the time course of the propagation phase and the concentration of the catechin used (Fig. 4). As shown in Fig. 4A, maximum acceleration was obtained when 2.0 μM EC was added 40 min after LDL oxidation, and the acceleration ratio was about 6 times. When the EC concentration was increased to 3.0–10.0 μM after 20 min, even in the propagation phase, EC inhibited LDL oxidation. The inhibition ratio was about −1.5 to −2.0, which means that TBARS values were lowered by EC additions. In the case of EGC (Fig. 4B), the maximum acceleration ratio was about 4 times. The maximum ratio was obtained with three different EGC concentrations (0.7, 1.5 and 2.0 μM) in each corresponding time course of the propagation phase. Addition of more than 1.5 μM EGC after the 20 min incubation period had a tendency to inhibit TBARS formation and the maximum inhibition ratio was about −2. The acceleration ratio of EC (6.0) was higher than that of EGC (4.0). Yet, the lower concentration of EGC accelerated TBARS formation more

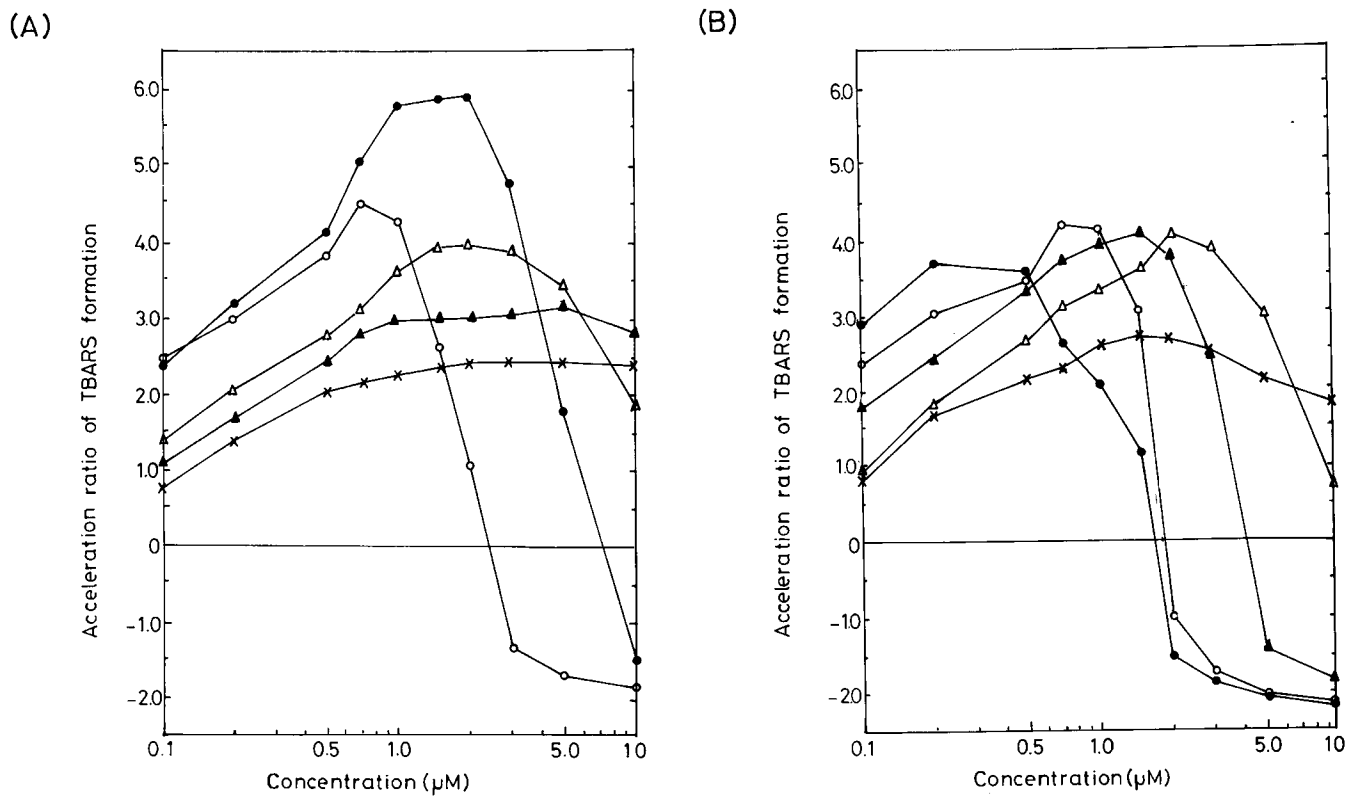


Fig. 4. Changes of acceleration and inhibition ratios by concentrations of EC or EGC added and time course of the propagation phase on LDL oxidation. LDL (0.2 mg protein/ml) was dissolved in 24 ml PBS-EDTA and the reaction was initiated with addition of 5 μM $CuSO_4$ at 37°C. Every 20 min (at 20, 40, 60, 80, and 100 min incubation), an aliquot (0.4 ml) was separated to add 0–10.0 μM EC (A) or EGC (B), and incubated for another 20 min. Then an aliquot (100 μl) from each reaction mixture was obtained to measure TBARS formation as described in Section 2. The acceleration or inhibition ratio at indicated time points after EC or EGC addition was calculated from each corresponding TBARS formation rate for 20 min incubation with EC or EGC addition against none. ○: 20 min; ●: 40 min; △: 60 min; ▲: 80 min; ×: 100 min.

effectively than those of EC (Fig. 4). The acceleration and inhibition of the oxidation of LDL in the propagation phase by EGC after 40 min incubation were also ascertained by measuring conjugated diene formation (Fig. 2B). The conjugated diene formation was accelerated by 1.5 μM EGC and inhibited by 5.0 μM EGC (Fig. 2B). As shown in Figs. 2B and 5, the effects of 5 μM NOC5 on Cu^{2+}-induced LDL oxidation were examined. In agreement with our previous paper [3], NO released from NOC5 inhibited LDL oxidation in the initiation and propagation phases. NO also clearly inhibited the 1.0 μM EC-induced accelerative LDL oxidation in the propagation phase as measured by TBARS formation (Fig. 5). The inhibitory effect of 1.0 μM EC in the initiation phase began to be released after 80 min and NOC5 addition inhibited the oxidation in the propagation phase (Fig. 5). The TBARS values were lowered when either EC or EGC was added to inhibit the LDL oxidation (−2 times) (Fig. 4A,B). Neither EGC nor NO lowered the values of conjugated diene formation (Fig. 2) and NO did not lower TBARS formation (Fig. 5). These facts suggest that catechins scavenged or inhibited chain reaction intermediates which might convert to TBARS.

4. Discussion

It is evident that EC, EGC and other catechins have inhibitory effects on Cu^{2+}-induced LDL oxidation in the initiation phase, in accordance with previous papers [1–3]. However, the effects of various catechins in the propagation phase of LDL oxidation have not been studied. In this paper, we found, by measuring TBARS formation, detecting conjugated diene and investigating fragmentation of apo B, that EC and EGC had both accelerative and inhibitory effects in the propagation phase of LDL oxidation depending on the concentration used and oxidation circumstances. Other catechins such as (+)C, ECG and EGCG also showed the same tendencies (data not shown). The accelerative and inhibitory effects of EC and EGC in the propagation phase of LDL oxidation were concentration and time dependent (Fig. 4A,B). However, in the initiation phase, even lower concentrations (5 nM–0.1 μM) of catechins did not accelerate oxidation but were inhibitory (data not shown). In the initiation phase, EC had a longer inhibitory effect of LDL oxidation than EGC (Fig. 1A,B). A higher concentration of EC than EGC was needed to accelerate or inhibit LDL oxidation. In order to obtain the maximum acceleration ratio in the time course of the propagation phase, concentrations of EC and EGC were increased depending on the oxidation state of the LDL and higher concentrations of either catechin were needed to inhibit the oxidation than to accelerate it (Fig. 4). The maximum acceleration ratio of EC was more than that of EGC. Yet, addition of EC had a longer inhibitory effect than addition of EGC (data not shown) as in the case of the initiation phase (Fig. 1). The above described antioxidative and oxidative facts indicate that catechins such as EC and EGC can be both oxidized and re-

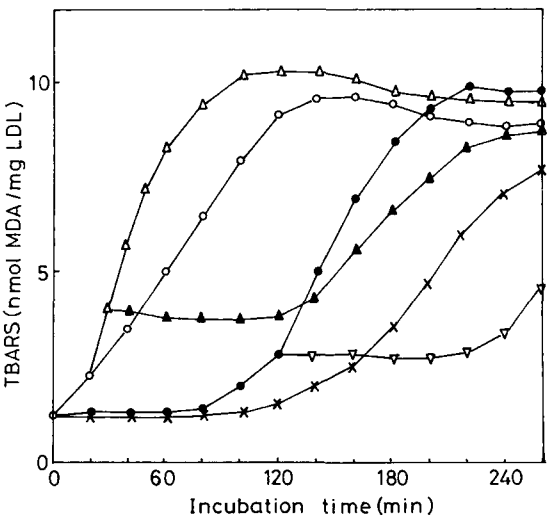


Fig. 5. Inhibitory effect of NO released from NOC5 on EC-accelerated Cu^{2+}-induced TBARS formation. LDL (0.2 mg protein/ml) was dissolved in 22 ml PBS-EDTA and the reaction was initiated with 5 μM $CuSO_4$ addition at 37°C, designated R.M. R.M. (6.0 ml) was continued to measure TBARS formation every 20 min interval as control (○). After 20 min incubation, R.M. (3.0 ml) was separated to add 1.5 μM EC (△). 10 min after EC addition, 5 μM NOC5 was added and an aliquot (100 μl) was removed every 20 min to measure TBARS formation (▲). In 6.0 ml of R.M., 1.5 μM EC was added (●) and after 120 min incubation, an aliquot (2.0 ml) was separated to add 5 μM NOC5 (▽). Every 20 min, an aliquot (100 μl) was removed to measure TBARS formation. R.M. (4.0 ml) was separated to add 5.0 μM NOC5 to measure TBARS formation (×). TBARS formation was measured as described in Section 2.

reduced through free radicals released by LDL oxidation. In the propagation phase, autoxidation of LDL might release burst amounts of various free radicals such as R•, RO•, ROO• and OH• [10,17] and oxidized EC or EGC might be re-reduced to accelerate the LDL oxidation depending on the oxidation and reduction states. In contrast to EC (Fig. 5) as well as EGC (Fig. 2B), NO inhibited LDL oxidation in both phases (Fig. 2B, Fig. 5). Therefore, the different electron transfer mechanism of antioxidative reagents such as catechins and NO must be related to oxidation reaction intermediates and the stability of oxidized forms of antioxidants. There are many natural flavonoid antioxidants in foods [17]. The anti-oxidative and oxidative character of catechins must be further studied for their appropriate use as well as toxicological implications. The results encourage us to investigate feasible conditions for uptake as foods and/or drinks.

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